Phosphorylation of c-Jun N-terminal kinase isoforms and their different roles in spinal cord dorsal horn and primary somatosensory cortex

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Abstract

The present study was undertaken to investigate whether isoforms of c-Jun N-terminal kinase (JNK 46 kDa and 54 kDa), one component of the mitogen-activated protein kinase (MAPK) family, might show region-related differential activation patterns in both naïve and pain-experiencing rats. In naïve rats, no significant difference was observed in total expression level of the two JNK isoforms between spinal cord and primary somatosensory cortex (S1 area). However, phosphorylated JNK 46 kDa was normally expressed in the S1 area, but not in the spinal cord, while neither of the two structures contained phosphorylated JNK 54 kDa. Subcutaneous bee venom (BV)-induced persistent pain stimulation resulted in a significant increase in the phosphorylation of both JNK isoforms in each area for a long period (lasting at least 48 h). Nevertheless, JNK 46 kDa exhibited a much higher activation than JNK 54 kDa in the spinal cord, whereas the same noxious stimulation elicited evident activation of JNK 54 kDa in the S1 area, leaving JNK 46 kDa less affected. Intraplantar injection of sterile saline solution, causing acute and transient pain, produced almost the same changes in activation profile of the two JNK isoforms as found in the BV-treated rats. These results implicate that individual members of the JNK family may be associated with specific regions of nociceptive processing. Also, the two JNK isoforms are supposed to function differently according to their locations within the rat central nervous system.

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It has been well defined that nociceptive information is transmitted from peripheral nociceptors through dorsal root ganglion to the spinal cord dorsal horn, where, after central processing, integrated pain signals are further ascended via thalamus to higher brain structures, such as the primary somatosensory cortex (S1 area) [9]. In spite of overwhelming evidence derived from human and animal studies showing active involvement of S1 area in the encoding process of noxious information (such as location, duration, quality and intensity coding of pain) [8,13,14,16], it remains less understood whether such higher level pain-associated brain structure employs the same or different signaling molecules in response to peripherally transmitted nociceptive signals in comparison with the spinal cord. Recently, our immunoblotting studies have demonstrated region-related differences in activation of extracellular signal-regulated kinase (ERK) isoforms, one component of the mitogen-activated protein kinase (MAPK) family, following peripheral noxious stimulation, implicating different molecular participants in pain processing at different levels [12]. Then it might be natural to ask whether isoforms of c-Jun N-terminal kinase (JNK 46 kDa and 54 kDa), another member of the MAPK family, might also have such characteristics in activation patterns under the pain state.

Therefore, in the present study, the bee venom (BV) test, a well-developed tonic pain model [4–6,15], was adopted to pro-
duce a kind of peripheral persistent pain stimulation to see the alterations of JNK isoforms activation in both the spinal cord and S1 area by using the Western blot technique. Our previous series of behavioral and electrophysiological experiments have established the idea that the BV model of inflammatory pain, compared with other traditional pain models such as the formalin test [1], may be more useful in elucidating both peripheral and central mechanisms of pathological pain as well as in screening new analgesic compounds [2,3,5–7,24,25]. In addition, based on the assumption that persistent pain differs from acute, transient pain in either clinical manifestations or pathophysiological mechanisms [4,22,23], we also try to provide an initial investigation into the region-related differential activation of JNK isoforms under the saline injection-induced transient pain state.

Experiments were carried out on male Sprague–Dawley rats (200–250 g; Animal Center of Capital Medical University, Beijing, PR China). Animals were housed two per cage and maintained on a 12 h light/dark cycle with food and water ad libitum. All experimental procedures were performed in accordance with ethical guidelines of the International Association for the Study of Pain for pain research in conscious animals [27] and were approved by Institutional Animal Care and Use Committee of Capital Medical University. As described elsewhere [6,15] a volume of 50 μl BV solution (2 μg/μl), lyophilized whole venom of Apis mellifera dissolved in 0.9% sterile saline, the crude venom of honeybee was obtained from Georgian gray bees kindly provided by Floret Ltd. and its partner company New Techniques Laboratory Ltd. (Tbilisi, Georgia) was subcutaneously injected into the posterior plantar surface of right hindpaws in conscious rats to produce a kind of persistent pain stimulation. Besides, similar injection of a sterile normal saline solution (pH = 6.4, Sodium Chloride Injection for medical use, purchased from Yangzhou Zhongbao Pharmaceutical Co., Ltd.) was performed to cause a transient pain in a parallel group of rats. Animals without any treatment were assigned as the naïve group.

For tissue collection and sample preparation, at indicated time points after subcutaneous saline or BV injection (5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 12 h, 24 h and 48 h), all rats (n = 3 for each group at each time point) were anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg, i.p.). Brains were rapidly removed after decapitation, briefly chilled on ice, and the contralateral S1 area (left-side part in this study) were rapidly removed after decapitation, briefly chilled on ice, and were stored at −20 °C. Protein content of lysates was determined using the bicinchoninic acid assay kit (Pierce, USA).

For Western blot analysis, proteins (30 μg/lane) were separated by SDS-PAGE on pre-cast 10% polyacrylamide gels. Following electrophoretic transfer onto nitrocellulose membranes, blots were incubated in blocking buffer (10% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20) for 1 h at room temperature (RT), washed 3 × 10 min in TTBS (20 mM Tris–Cl, pH 7.5, containing 0.15 M NaCl and 0.05% Tween 20) and incubated with primary phosphospecific antibody (rabbit anti-mouse p-JNK 46 kDa/54 kDa pAb, Cell Signaling Technology, USA) at a 1:1000 dilution for 4 h at RT. Blots were further washed three times (each for 10 min) in TTBS, incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Bio-Rad, USA) at a 1:3000 dilution for 1 h at RT, washed again, treated with ECL reagent and exposed to film. Then blots were stripped by incubation for 15 min at 50 °C with the stripping buffer, re-blocked, washed and incubated for 4 h at RT with the corresponding pan antibody which recognized total antigen protein (1:1000; rabbit anti-mouse pan-JNK 46 kDa/54 kDa pAb, Cell Signaling Technology, USA). Subsequent procedures were the same as described above.

Immunoblotting against β-actin, a stably expressed protein, was finally performed as the internal control. The experimental protocols were almost identical with those mentioned above except that the primary antibody was mouse anti-human β-actin mAb (Sigma, USA) and the second antibody was of goat anti-mouse source (Bio-Rad, USA).

A computerized image analysis system (GelDoc-2000 Imagine System, Bio-Rad, USA) was utilized to analyze immunoreactive bands as described previously [18]. For each blot, the relative value of phosphorylation level was calculated from the ratio of absorbance of the phospho-protein/pan protein to correct for small differences in protein loading, while the relative density of each band corresponding to pan protein of JNK was normalized to the value of β-actin band to illustrate the protein expression level. All results were expressed as mean ± S.E.M. Statistical analysis was carried out with one-way ANOVA followed by individual post hoc multiple comparisons (Fisher’s PLSD test). P < 0.05 was considered statistically significant.

As can be readily seen from the original immunoblotting bands, both JNK isoforms (JNK 46 kDa and 54 kDa) were constitutively expressed in the normal spinal cord and S1 area (Fig. 1A and B). Statistical analysis found no significant difference in total expression of JNKs between the two regions. In the case of phosphorylated JNks, however, dramatic differences were observed in the immunoreactivity of phospho-JNK 46 kDa between the spinal cord and S1 area, with the latter showing much stronger band density than the former (Fig. 1A and B). Quantitatively, statistical analysis further confirmed this phenomenon (S1 area versus spinal dorsal horn: P = 0.003 < 0.01, n = 3, Fig. 1C). No regional differences were detected in normal expression of phospho-JNK 54 kDa (Fig. 1A, B and D).

Experimental persistent pain stimulation produced by s.c. injection of BV solution resulted in an apparent increase in
phospho-JNK protein levels in both ipsilateral spinal cord and contralateral S1 area (Fig. 1A and B). Total JNK expression levels were unaltered by noxious stimulation, indicating that the observed increase in phospho-JNK level was caused by enhanced phosphorylation but not elevated substrates. Further, the time course study demonstrated long-term activation of both JNK isoforms by BV-induced persistent pain in each area, which started from 5 min after intraplantar treatment, peaked at about 1–2 h but maintained at a high level even at 48 h after s.c. BV injection (data not shown). Interestingly, intraplantar injection of sterile saline solution, eliciting a form of acute and transient pain, also led to a similar activation of JNKs in both spinal cord and S1 area (Fig. 1A and B) and the duration of this saline-evoked JNKs phosphorylation was no shorter than that caused by s.c. BV injection (data not shown).

In spite of the generally increased activation of JNKs by painful stimulation (transient or persistent), the present immunoblotting data also revealed a marked regional difference in the response intensity of the two JNK isoforms. In the spinal cord, although both JNK isoforms were phosphorylated by s.c. saline or BV injection, the level of phospho-JNK 46 kDa was clearly higher than that of phospho-JNK 54 kDa (Fig. 1A). By contrast, within the S1 area, phospho-JNK 54 kDa level was obviously upregulated in either saline- or BV-treated rats, whereas the phosphorylation level of JNK 46 kDa was less changed, perhaps due to its high basal expression under the naïve state (Fig. 1B). A quantitative analysis of the summarized data was illustrated in Fig. 1C and D. Statistically significant differences were found in phosphorylation level of JNK 46 kDa in the spinal cord (BV-treated versus naïve rats, $P = 0.005 < 0.01$, saline-treated versus naïve rats, $P = 0.02 < 0.05$; $n = 3$, Fig. 1C) with JNK 54 kDa being less influenced (BV-treated versus naïve rats, $P = 0.07 > 0.05$, saline-treated versus naïve rats, $P = 0.08 > 0.05$; $n = 3$, Fig. 1D). By analogy, there also existed a significant difference in the band density of phospho-JNK 54 kDa in the S1 area (BV-treated versus naïve rats, $P = 0.006 < 0.01$, saline-treated versus naïve rats, $P = 0.005 < 0.01$; $n = 3$, Fig. 1D), whereas the phosphorylation level of JNK 46 kDa was unaltered (BV-treated versus naïve rats, $P = 0.08 > 0.05$, saline-treated versus naïve rats, $P = 0.06 > 0.05$; $n = 3$, Fig. 1C). Another critical discovery of the present results was that in saline-treated animals, the activation profile of the two JNK isoforms was almost identical with that in the BV-inflamed animals (also see Fig. 1C and D). No significant differences were found between the transient pain and the persistent pain group.

In the present study, tissue homogenates taken from either spinal cord or S1 area were subjected to Western blot analysis and two immunoreactive bands were finally detected in the membrane with the molecular weight of 46 kDa and 54 kDa, respectively (Fig. 1A and B). Furthermore, the two JNK isoforms were highly expressed in both spinal cord and S1 area of cortex.
in naïve rats, suggesting that the JNK protein was extensively distributed in the rat central nervous system (CNS) under the normal state, which was in accordance with one previous study showing moderate to intense staining of JNK1 and MEK4 (one of its upstream activators) in layers II–VI of the neocortex and light to moderate staining of these two kinases in the spinal cord [10]. This basal widespread distribution profile of JNKs might lead us to the hypothesis that JNK, as one of the most important intracellular signal transduction mediators and effectors, is constitutively and ubiquitously expressed in the rat CNS in normal conditions, behaving as a fundamental integrator of multiple inputs originating from diverse sources. However, it should be important to note that no significant regional differences were found in the expression level of the two JNK isoforms, which was in contrast to the normal distribution of ERK isoforms as reported previously [12].

One of the most interesting aspects of the present findings was that phospho-JNK 46 kDa was normally expressed with a high level in the S1 area, but not in the spinal cord (Fig. 1A–C). However, neither of the two structures was found to contain phosphorylated JNK 54 kDa in the naïve conditions (Fig. 1A, B and D). Heretofore, there is no information regarding the exact mechanisms underlying this basal activation of the JNK 46 kDa within the S1 area of cortex. Speculatively, we presume that it might have some causal relationships with the frequent touch onto the ground of the home cage by rat paws under normal, unstimulated state. It is reasonable to believe that rats without any insult will stand and move on the cage ground frequently. In this case, existing tactile stimuli can also activate certain cortical domains in the S1 area of cortex (including area 3b and area 1), as reported previously [11,20]. As a consequence, it appears likely that this cortical processing of tactile stimuli makes the pJNK 46 kDa inducible in naïve rats. It would be interesting in future studies to determine whether there are similar changes in phosphorylation of JNKs in other regions associated with tactile signal transmission, such as posterior funiculus, gracile/cuneate nucleus and so on.

In the present experiments, we also explored whether or, if so, to what extent peripherally induced transient or persistent pain stimulation will influence JNKs phosphorylation status and their distribution patterns in the spinal cord and S1 area of cortex. Our observations strongly indicated a long-term (lasting at least 48 h) phosphorylation (activation) of both JNK isoforms in the two regions under either transient or persistent pain state, without affecting the total expression level of the JNK proteins. That BV-produced inflammatory pain significantly elevated spinal phospho-JNK level is in line with other past reports demonstrating similar activation of JNKs in the spinal dorsal horn astrocytes following spinal nerve ligation [26] or partial sciatic nerve ligation [17], two models of neuropathic pain. According to those studies, it might be instructive, in a future study, to explore the cell-type-dependent distribution and subcellular localization of activated JNK isoforms in the BV model by using immunocytochemical staining technique. Curiously, the duration of spinal JNKs activation triggered by nerve injury in those studies (>21 days) is much longer than that initiated by tissue injury in this study (>2 days). The discrepancy is, on our hand, mainly due to different animal models used, because it is clear that the time course of pain-related behavioral responses (nociception, hyperalgesia or allodynia) may differ substantially from each other in different animal models of pathological pain. With respect to the S1 area, our new data showing pain-enhanced JNKs activation in this region, for the first time, might set foundations for future research into the possible roles of JNK-associated signaling mechanisms in the nociceptive signal processing of higher brain structures. Nonetheless, the nature of the extracellular signaling molecules that were recruited upon subjecting animals to painful stimulation and hence responsible for JNKs activation was still not clear. Additional studies will be required to answer this question.

There is general agreement that persistent or chronic pain differs greatly from acute or transient pain in terms of etiology, symptom, mechanisms and pathogenesis [4,22,23]. Therefore, a difference in the intracellular signaling mechanisms of the two pain states might be naturally expected. Surprisingly, the present immunoblotting results did not reveal any significant differences in the activation of JNK 46 kDa or 54 kDa in both regions between saline- and bee venom-treated rats with regards to either response intensity or duration. The precise mechanisms for this saline-induced long-lasting phosphorylation of JNKs remain less understood until now. However, in one previous study, we also found such saline-elicited prolonged activation of ERKs in both spinal cord and S1 area [12]. Thus, as discussed in that paper, it is speculated that the low pH nature of the saline solution used in the current study might be responsible, at least in part, for this phenomenon.

Another most crucial discovery of the present work was that regional selectivity in the activation magnitudes of the two JNK isoforms was detected following peripheral noxious stimulation (either saline-induced transient pain or BV-induced persistent pain) (see Fig. 1C and D). While spinal JNK 46 kDa exhibited much stronger activation than JNK 54 kDa, in the S1 area, phospho-JNK 54 kDa was predominantly enhanced compared with the phospho-JNK 46 kDa. This region-associated differential activation of JNK isoforms is in parallel with our previous ERK immunoblotting findings, which also showed robust regional differences in activation of ERK1 and ERK2 following s.c. BV injection [12]. An important implication of these data is that specific ERK or JNK members are dedicated to playing different roles in the nociceptive processing occurring at different levels of pain ascending pathways. The exact reasons for functional differences between the two JNK isoforms in mediating pain processing were not fully recognized. Presumably, structural differences and distinct in vitro biochemical properties between them might be partially attributable [21].

In conclusion, the present study has contributed the new finding that either transient or persistent pain stimulation can effectively drive a long-term activation of the JNK signaling pathway, but the response patterns differed greatly between specific JNK isoforms in different regions. In the spinal cord, phosphorylated JNK 46 kDa is likely to mainly participate in the nociceptive signal transmission and modulation, while in the S1 area, it is phosphorylated JNK 54 kDa that might play essential roles in the higher level of pain processing. Additionally, the
current results showing normal activation of the JNK 46 kDa isoform in the S1 area may shed new insights into the participation of the JNK signaling family in the non-painful perception.

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